Effects of sleep deprivation on the development of autoimmune disease in an experimental model of systemic lupus erythematosus

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Sleep is hypothesized to play a restorative role on immune system. In addition, disturbed sleep is thought to impair host defense mechanisms. Chronic sleep deprivation is a common occurrence in modern society and has been observed in a number of chronic inflammatory conditions, such as systemic lupus erythematosus (SLE). New Zealand Black/New Zealand White (NZB/NZW) F1 mice develop an autoimmune disease that strongly resembles SLE in humans, exhibiting high titers of antinuclear antibodies associated with the development of rapidly progressive and lethal glomerulonephritis. On the basis of this evidence, the present study examined the onset and progression of lupus in as-yet healthy female mice submitted to sleep deprivation. Sleep deprivation was accomplished by two 96-h periods in the multiple-platform method when mice were 10 wk old, and they were observed until 28 wk of age. Blood samples were collected from the orbital plexus fortnightly to evaluate serum antinuclear antibodies and anti-double-stranded DNA. Proteinuria and longevity as well as body weight were also assessed. The results indicated that mice submitted to sleep deprivation exhibited an earlier onset of the disease, as reflected by the increased number of antinuclear antibodies. However, no statistical difference was found in the other parameters analyzed. According to these results, sleep deprivation could be considered as a risk factor for the onset but not for the evolution of the disease.

Increasing evidence suggests a bidirectional communication between sleep and the immune system. On one hand, mediators of the immune response regulate physiological sleep, both at normal and exacerbated function, which can be observed during infection (for reviews, see Refs. 2 and 34). On the other hand, sleep is hypothesized to have a restorative function on immune processes. On the basis of results from a study (13) in which sleep deprivation (SD) caused the invasion of normally sterile body tissues by pathogenic bacteria, it may be considered that abnormalities of host defense including immune suppression would render healthy sleep-deprived individuals susceptible to disease. However, these relationships have not been studied extensively.

The hypothesis that sleep might influence host defenses has been suggested by the association between decreased sleep time and increased morbidity and mortality in humans (21, 61) and by the findings in rats that SD results in sepsis and eventually death (13). SD affects several other facets of the immune system, including antigen uptake (6), phagocytosis (37), mitogen responses (32), antibody responses (23, 49), and a variety of lymphocyte subsets (9).

The effects of SD on the immune system have been widely studied. This is an important issue in its own right because SD is becoming a more frequent and more accepted occurrence in modern society. In addition, sleep disorders have been observed in a number of chronic inflammatory conditions, such as autoimmune diseases. Some of these clinical states associated with sleep disturbances include rheumatoid arthritis, fibromyalgia, and systemic lupus erythematosus (SLE) (for a review, see Ref. 24). The sleep disruption that is observed in some of the immune and autoimmune disorders is most likely the result of dysregulations of the sleep-immune system relationship that merely a discomfort induced by the disease symptoms or manifestations, such as pain.

SLE is an autoimmune disease primarily affecting young women at reproductive age. The female-to-male incidence ratio is approximately 9:1 in most series (43). The preponderance of SLE in women may result, in part, from stimulation of the immune system by female hormones (28). This chronic inflammatory disease is characterized by the formation of autoantibodies and deposition of immune complexes, resulting in glomerulonephritis and vasculitis. In SLE, one of the dominant antibodies is directed against the cell nucleus: antinuclear antibodies (ANA). Estrogen, pregnancy, stress, and intake of a variety of drugs are associated with the onset and worsening of SLE (19, 35, 39, 42). Lupus patients complain of unrefreshing sleep and daytime fatigue, which results from sleep disturbances (8, 27, 53, 54, 56). According to Valencia-Flores and colleagues (56), these patients are sleepier during the day by virtue of sleep fragmentation due to more arousals and stage transitions. In addition, the disease is exacerbated by sleep disruption, resulting in decreased sleep efficiency and delta sleep.

New Zealand Black/New Zealand White (NZB/NZW) F1 mice spontaneously develop an autoimmune disease that closely resembles immunological and clinical characteristics of human SLE. The evolution of the disease is characterized by abnormal polyclonal B cell activation with a high production of ANA that includes anti-double-stranded DNA (anti-DNA), lymphadenopathy, arthritis, hemolytic anemia, vasculitis, and a variety of histopathological manifestations, of which glomerulonephritis is the most prominent (55). Also, similar to char-

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acteristics in humans, the disease is most frequent in female mice, and the susceptibility is influenced by the major histocompatibility complex (57).

Although a large number of studies have focused on the relationship between the immune system and SD, relatively few have examined the long-term effects of SD and/or have evaluated more than one postdeprivation recovery day to determine the time course of postdeprivation changes in immunological parameters. If one considers that increased sleep fragmentation and increased disease activity and pain sensitivity are common features of SLE patients, it is clearly necessary to investigate experimental paradigms that allow a more precise understanding of the impact of SD on autoimmune disease. To determine whether SD can precipitate or exacerbate the development of autoimmunity in genetically predisposed animals, the disease course in NZB/NZW F1 mice was assessed by following the development of ANA, proteinuria, and longevity.

MATERIALS AND METHODS

Animals and experimental procedures. All procedures were carried out in accordance with the guidelines on animal care of the National Institutes of Health and were approved by the Animal Care and Use Committee of the Universidade Federal de São Paulo (CEP No. 1163/01).

NZB (females) and NZW (males) mice were obtained from the Universidade de São Paulo (São Paulo, Brazil) and were mated in our Research Laboratory to produce NZB/NZW F1 hybrids. After being weaned, NZB/NZW F1 mice were housed in groups of six in plastic cages filled with hardwood bedding and provided water and rodent chow ad libitum. Animals were kept in a room with controlled lighting (12:12-h light-dark cycle) and temperature (24 ± 2°C). Because of the fact that murine lupus is more prevalent in females, only this gender was used in the present study. In addition, both control nondeprived and SD female mice were age matched because there is a natural age-dependent evolution of the disease.

SD procedures. Female NZB/NZW F1 mice aged 10 wk (an age in which they are clinically healthy) were subjected to SD using an adaptation of the multiple-platform method originally developed for rats (52). The technique is based on the muscle atonia that accompanies paradoxical sleep (18). Briefly, 12 narrow circular platforms (3 cm in diameter) were placed inside a tiled tank (41 × 34 × 17 cm) filled with water to within 1 cm below the upper border of the platform. Groups of six animals were placed on the platforms in each tank, an arrangement that allowed them to move inside the tank, jumping from one platform to the other. In this procedure, animals are aroused from sleep when the loss of muscle tone leads them to fall off the platform. This method produces a consistent amount of sleep reduction in mice (47).

Animals were randomly distributed into two groups: control mice, which remained in their home cages in the SD room (n = 22); and SD mice, which were deprived of sleep for two periods of 96 h each with an interval of 3 days (n = 28). This experimental protocol was carried out as an attempt to simulate a chronic condition of SD (similar to what is observed in chronic inflammatory disease). During the interval, animals were placed back in their home cages. Throughout the study, both groups had free access to food and water. After the end of SD, animals in the experimental group were placed back in their home cages, and both groups were observed for several weeks.

Blood sampling procedures and ANA determination. At 10 (before SD), 13, 16, 19, 22, 25, and 28 wk of age, mice were weighed, rapidly anesthetized (ether vapors), and bled from the orbital venous plexus with the use of plain capillary tubes (41). After being centrifuged, the serum was separated and stored at −20°C until analysis.

Determination of ANA and anti-DNA. ANA was determined by a standard indirect immunofluorescence (IIF) technique using Hep-2 cells as the substrate (Kallestad, Bio-Rad Laboratories, Redmond, WA). IIF on Hep-2 cells remains the method of choice for ANA detection (48). In the case of negative ANA, the diagnosis of SLE is very unlikely. The manufacturer’s protocol was followed. Briefly, autoantibodies in a test sample bound to antigens in the substrate. A wash with PBS removed the excess serum from the substrate. Fluorescein-conjugated (FITC) antiserum added to the substrate attached to the bound autoantibody. FITC-conjugated rabbit antibodies against mouse IgG were kindly donated by Biolab. In these procedures, a fluorescent antibody served as the marker for the antigen-antibody binding reaction, which occurred on a substrate surface. After a second wash step to remove the excess conjugate, the substrate was coverslipped and checked for fluorescent patterns with a fluorescent microscope. Observation of a specific fluorescent pattern on the substrate indicated the presence of autoantibodies in the test samples. A positive and negative control was included with each assay run. Sera were considered positive for the presence of ANA at a starting dilution of 1/50.

All positive sera were tested for anti-DNA antibodies by IIF on Crithidia luciliae as a substrate (DTS) following the same procedures used for ANA detection.

Measurement of proteinuria. To evaluate the severity of the autoimmune disease, proteinuria, a surrogate marker of autoimmune nephritis, was performed. At 24, 31, and 37 wk of age, mice were placed in metabolic cages, and urine was collected. The protein concentration was determined by precipitation with 3% sulfosalicylic acid in 24-h urine samples, and measurements were performed by spectrophotometry (Spectronic, Genesys 5 Milton Roy) (15). Spectrophotometric measurement was carried out with a microplate reader at 650 nm. To convert optical density values, a standard curve was generated for each assay.

Longevity study. Mice were examined daily for signs of disease (edema, lethargy, anorexia, pilorection, or rough hair coats) and death.

Data analysis. The Kaplan-Meier estimator was used to estimate the proportion of the number of mice that produced ANA and also to estimate the proportion surviving (life span) by given time. Mice that did not produce ANA or did not die until the end of the study were considered censored data. Data containing uncertainty as to when exactly an event happened are termed as censored data. Proportion and survival curves were compared using the log rank test. Visual inspection revealed differences between weeks 10 and 13 that might be relevant, although not sufficiently to provide statistical evidence by the log rank test for analysis of the whole period of study. Therefore, the binomial test for proportions was used to analyze ANA production in each week, independently. Statistical analysis of the anti-DNA data was performed with the binomial test for proportions. The remainder of the data was compared by two-way repeated-measures ANOVA followed by the Tukey test for unbalanced groups. All data are expressed as means ± SE. Statistical significance was set at P ≤ 0.05.

RESULTS

ANA and anti-DNA. Log rank data revealed that proportion curves were similar between control and SD groups (P = 0.4185), indicating that ANA production was similar between the groups (Fig. 1). Nonetheless, no new case was observed between the 10th and 13th week in control mice, meaning a 0% transition. In the SD group, however, five new cases were found during the same time interval, resulting in 21% transition. The binomial test showed a statistical difference between control and SD groups (0% × 21%, P < 0.03). No further differences were revealed throughout the study. The production of anti-DNA across both groups indicates that the antibody
is spontaneously produced in this mouse strain and is not influenced by SD.

**Body weight.** Analysis of the data considering the body weights immediately before and immediately after SD (10th vs. 12th week of life) showed a main effect of time \( F(1,19) = 9.87 \) and \( P = 0.005 \) but no effect of group or an interaction between these factors. The post hoc analysis indicated that both groups gained weight immediately after the period of SD (week 12) compared with week 10 (Fig. 2). Analysis of body weights throughout the study period again revealed a main effect of time \( F(6,162) = 46.17 \) and \( P < 0.0001 \), but no interaction between group and time. The test of Tukey showed that, for both groups, weight gain was greater on the 16th week than on the previous weeks of life \( (P < 0.0001) \); Fig. 3).

**Proteinuria.** In terms of proteinuria, again, a main effect of time was detected \( F(2,32) = 51.03 \) and \( P < 0.0001 \). In this case, augmented protein excretion was observed on the 37th week of life in both groups compared with the previous time points \( (P < 0.0001); \) Fig. 4.

**Life span.** The log rank test did not detect a difference between the survival curves of the groups \( (P = 0.1317) \), and the average life span was 38 and 40 wk for the control and SD groups, respectively (Fig. 5).

**DISCUSSION**

According to the present findings, SD produced an immediate and short-lasting effect, manifested by an earlier triggering of the disease, which was reflected by more cases of positive ANA. Nonetheless, SD did not affect the evolution or severity of the disease, according to the proteinuria and longevity data.

Similar to other studies (7, 25), the onset and evolution of the disease was based on the presence and amount of ANA. This is an extremely sensitive and specific parameter, and, contrary to human beings, it is a measurement of disease activity.

One of the most replicable effects of SD in rats is the progressive increase of energy expenditure, manifested by augmented food intake and weight loss (3, 11, 16). Contrary to what is observed in rats, SD did not produce weight loss in NZB/NZW F1 mice. Both control and SD mice gained weight after the manipulation compared with their pre-SD body weight. Although we did not assess food intake, it could be hypothesized that this distinct effect of SD is due to differences in basal metabolism between rats and mice. The assessment of body weight throughout the disease evolution showed that both groups gained weight, mainly after the 22nd week of life.
is likely due to a generalized edema resulting from glomerulonephritis (55).

Glomerulonephritis is the most common kidney impairment in sick animals and the most important cause of death (55), and protein loss in the urine is the best evidence of such an impairment. In the present study, there was a progressive loss of protein in the urine for both groups, with a distinct increase on the 37th week, reaching average levels of 37 mg/day, i.e., a 10-fold increase. This finding is in agreement with the literature, which indicates a massive protein loss from the eighth month of life on (26, 55).

The average life span was similar for both groups, 38 and 40 wk for control and SD groups, respectively. This result is in accordance with an extensive review published by Theofilo-poulos and Dixon (55), who reported the survival rate of several mouse strains of lupus. For NZB/NZW F1 mice, the survival rate of females, which exhibit accelerating factors such as high ANA titers and the presence of anti-DNA antibodies, varied from 36 to 39 wk. Anti-DNA antibodies production in the NZB/NZW F1 model occurs later in life, approximately between the fourth and fifth months of age, with the highest prevalence on the ninth month, and rarely before the second month of life. Even though this is seldom, some cases can be found in the early period (55). These antibodies are particularly important due to their nephritogenic properties. An immunofluorescence and kidney eluate study (22) has indicated that DNA and its antibody of the IgG class are deposited in the kidney. Moreover, there is a significant correlation between the onset of clinical symptoms and switch from IgM to IgG, and this alteration is more pronounced in females (38, 50).

The present results showed that SD accelerated the production of circulating ANA. The mechanisms by which SD affects the immune system are not completely understood, but systems that regulate immune activity are also affected by SD, such as the endocrine system. For instance, Everson and Crowley (12) believe that the reduction of immune activity after SD is due to decreased secretion of growth hormone (GH) and prolactin (PRL). Lange and colleagues (23) observed that SD subjects who exhibited low production of antibodies also showed low GH and PRL levels. Therefore, PRL appears to be important for the integrity of the immune system. Several studies using SLE attribute to PRL a disease-triggering or -stimulating effect. There is a positive correlation between PRL concentrations and the increase of anti-DNA antibodies (33). In NZB/NZW F1 mice, PRL is immune stimulating, and modest elevations of blood concentrations lead to hyper-γ-globulinemia and an augmented production of immune complexes, resulting in glomerulonephritis and, ultimately, death (30). In addition, female mice whose PRL secretion is inhibited by treatment with bromocriptine display a postponed onset of SLE (29).

Although the literature indicates that SD reduces PRL secretion, there is no report on its effect in NZB/NZW F1 mice; nonetheless, PRL seems to be deleterious to these mice.

Some studies have indicated that sleep loss alters immune response-related parameters, such as cytokine levels. Recently, a study (17) showed that SD induced the secretion of inflammatory cytokines, including IL-1, TNF, and IL-6. Asreviewed by Kishimoto and Hirano (20), abnormal IL-6 production results in polyclonal B cell activation and the occurrence of autoimmune features. IL-6 has been implicated in the pathogenesis of murine lupus, because NZB/NZW F1 mice exhibit elevated levels of this cytokine, and their lymphocytes are also hyperresponsive to IL-6 (1). In addition, Mihara and coworkers (31) have shown that blockade of IL-6 receptors inhibited the development of the disease in these mice and that the administration of recombinant IL-6 exacerbated glomerulonephritis (44). Given that this cytokine is harmful to SLE, it is possible to speculate that SD induced an increase in IL-6 levels in NZB/NZW F1 mice, resulting in an earlier onset of the disease. Another interesting finding that might explain the increased production of ANA, which was reported by Everson (10), is that clinical immune parameters measured after SD in rats suggest the activation of mechanisms associated with innate immunity and responses by B lymphocytes that are consistent with polyclonal activation. Various reports (14, 46, 58, 60) have shown that the acceleration of autoimmune disease is associated with polyclonal activation of B cells. On the basis of these studies together with the present results, we do not believe that SD can be conceptualized as being only immunosuppressive condition. It is difficult to draw any firm conclusions about the effects of SD on the immune system. SD involves, to variable degrees, imposition of nonspecific stress, which may interact with the effects attributable to sleep loss per se. Discrepancies in literature may be related at least in part to technical approaches and the duration of SD. It is difficult to compare results of different deprivation protocols and durations, but it is known that the principal approaches used in the literature (disk over water, platform, and gentle handling) in fact induce SD. Despite these limitations, there is emerging evidence that in rodents chronic sleep loss is detrimental rather than acute sleep loss.

Some studies (5, 39, 59) have suggested that high levels of life stress precede the disease outbreak or exacerbation in patients with SLE. In an experimental model, Chida et al. (7) demonstrated that social isolation stress exacerbates autoimmune disease in MRL/lpr mice. However, the potential physiological mechanisms that could justify such an association remain to be clarified. It is difficult to determine exactly which pathway is being altered by stress. Even if it is an immune pathway, it must be altered in a direction that will lead to disease onset or exacerbation. Several lines of evidence support the notion that SD is a stressful stimulus. Both ACTH and
corticosterone levels are increased after SD (36, 45, 51). Thus, it is tempting to speculate that the increased ANA production observed after SD may be mediated by activation of the hypothalamic-pituitary-adrenal axis. Although the exact mechanism of the stressor-induced increased susceptibility to the development of an autoimmune disease is not known, some possible explanations can be offered. As reviewed by Rabin (40), it is possible that stress hormones alter the kinetics of maturation of B lymphocytes so that there is a shorter window of opportunity for B lymphocytes to become tolerant to self-antigens. Besides, it is possible that the hormonal responses to stress alter the interactions between the cells that are responsible for the induction of tolerance of T lymphocytes, resulting in an increased number of autoreactive T cells.

Several studies using different deprivation paradigms have shown significant alterations in immune markers in response to SD. Such data do not necessarily provide an indicator of how SD impacts on immune function and how impaired is an organism. However, recent important studies evaluated the functional integrity of the immune system in a SD condition. Spiegel et al. (49) and Lange et al. (23) have reported that the response to influenza or hepatitis A vaccination may be impaired in individuals with chronic sleep restriction. Such data are so relevant, inasmuch as the antibody response to vaccina
tion constitutes a valid tool to assess the impact of SD on adaptive immune functioning. In the same way, the present results demonstrate clearly that the SD accelerated the timing of autoantibody production; thus, sleep loss affects host outcome and may be harmful to the host. This study provides valuable information regarding how sleep loss can impact the autoimmune disease process.

In conclusion, the present data indicate that SD is capable of accelerating the onset of lupus in NZB/NZW F1 mice, consid-
ering ANA production as the end point. This result emphasizes the importance of adequate sleep in SLE, in which chronic sleep disturbances are reported. Despite the fact that SD exerted a triggering effect, it did not alter the evolution or severity of the disease, suggesting an acute impact. Drawing of such a conclusion was only possible because we performed a prolonged followup observation of SD effects on the immune system; most studies have assessed only the acute effects, which may be misleading in respect of the impact of sleep loss on autoimmune diseases.

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REFERENCES


